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Effective Immunity to Dental Caries: Dose-Dependent Studies of Secretory Immunity by Oral Administration of *Streptococcus mutans* to Rats

SUZANNE M. MICHALEK, JERRY R. McGHEE,* AND JAMES L. BABB

Department of Microbiology and The Institute of Dental Research, University of Alabama in Birmingham, University Station, Birmingham, Alabama 35294

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Rats (COBS/CD) provided Formalin-killed *Streptococcus mutans* 6715, C211 in their drinking water (10^8 to 10^9 equivalent colony-forming units [CFU] per ml) had high levels of specific antibodies in saliva, colostrum, and milk. Rats provided a lower concentration of *S. mutans* antigen (10^7 CFU per ml) in water had agglutinin titers in secretions that were similar to those in controls. Gnotobiotic rats provided *S. mutans* antigen in food (10^7 to 10^8 equivalent CFU per g of diet) manifested a secretory immune response as evidenced by the presence of specific immunoglobulin A antibodies in saliva, colostrum, and milk. Gnotobiotic rats provided a higher concentration of antigen (10^9 CFU per g) in food had levels of specific antibodies in their secretions that were similar to those in controls. No significant antibody activity to *S. mutans* was observed in sera of any group of animals. Furthermore, the presence of specific salivary immunoglobulin A antibodies in gnotobiotic rats correlated with a reduction in the level of plaque, numbers of viable *S. mutans* in plaque, and levels of *S. mutans*-induced dental caries. This paper discusses the importance of antigen dosage for induction of a secretory immune response that is protective against *S. mutans*-induced dental caries.

Dental caries is perhaps the most prevalent infectious disease afflicting humans today (32). *Streptococcus mutans* has been implicated as a principal etiological agent of dental caries in humans (9, 16) and experimental animals (14, 15, 23) because this oral bacterium has the ability to adhere to and produce acid at the tooth surface when certain carbohydrates, especially sucrose, serve as a substrate (9, 36). However, other oral bacterial species probably contribute to this disease (4, 8). Recent investigations have demonstrated that local injection followed by direct instillation of *S. mutans* antigen into the parotid duct of monkeys induced the production of secretory immunoglobulin A (s-IgA) antibodies to *S. mutans* (6). The presence of these antibodies correlated with a marked reduction in the number of infected sites and the numbers of *S. mutans* on infected tooth surfaces (7). Results from experimental rodent studies have demonstrated that injection, in the salivary gland region, of either whole, killed *S. mutans* (18, 34) or glucosyltransferase enzyme preparations of *S. mutans* (34) induced a local secretory immune response. The presence of salivary s-IgA antibodies correlated with a reduced incidence in caries (18, 33, 34). These findings suggest an important role for s-IgA antibodies in the control of dental caries.

Other investigations have demonstrated that oral or intragastric administration of antigens such as erythrocytes (1), viruses (29), or bacteria (12, 22) results in the appearance of antibodies in external secretions (including saliva) that are predominantly of the s-IgA class (11). The simultaneous appearance of these s-IgA antibodies in external secretions remote from the site of stimulation poses a question as to the origin of the lymphoid cells responsible for this activity. Recently reviewed (30) studies from several laboratories offer a possible explanation. Briefly, this involves antigenic stimulation of lymphoid cells that are located in gut-associated lymphoid tissue. These stimulated lymphoid cells leave the gut-associated lymphoid tissue and migrate through the lymphatics via the mesenteric lymph nodes and enter the blood stream. From the circulation system, these cells home to secretory tissues, including mammary, salivary, and lacrymal glands as well as the lamina propria of the gastrointestinal and respiratory tracts. In the environment of these tissues, these lymphocytes further differentiate into mature immunoglobulin A (IgA)-secreting plasma cells

with antibody specificity to the ingested antigen.

These recent studies indicate the induction of a selective secretory immune response after oral ingestion of antigen (10, 19a, 22, 27); however, little information is available concerning the influence of the amount of antigen given on the selective induction of a secretory immune response. Recent results by André and co-workers (2) have indicated that intragastric immunization of mice with erythrocyte antigen can result in a condition of hyporesponsiveness; this observation suggests that the induction of immunological tolerance may have occurred. We report here that oral administration of various doses of *S. mutans* antigen to rats induces a selective secretory immune response.

MATERIALS AND METHODS

Animals. Two strains of rats were used in this study—(i) Sprague-Dawley-derived COBS/CD and (ii) germfree Fischer CD F(344)GN (original breeding colonies obtained from Charles River Breeding Laboratories, Inc., Wilmington, Mass.). The original stock of COBS/CD rats was treated with antibiotics (21) and maintained in a horizontal, double-sided, laminar air-flow unit (Germfree Laboratories, Inc., Miami, Fla.). This colony of rats is free of all indigenous cariogenic bacteria. The COBS/CD rats were housed in sterile plastic cages containing autoclaved hardwood laboratory bedding (Northeastern Products Corp., Warrensbury, N.Y.) in a room maintained at a constant temperature (25 ± 2°C), 50% relative humidity, and a 12-h light cycle. The germfree rats were maintained under similar housing conditions in Trexler plastic isolators (24, 35). Adult breeding rats were provided autoclaved Wayne Lab-Blox sterilizable, fluoride-free, rat chow (Allied Mills, Inc., Chicago, Ill.) and sterile drinking water ad libitum. After parturition, eight to nine pups were assigned to each nursing dam.

Microbiology and immunogen preparation. In this study a mutant of *S. mutans* 6715 wild type, designated as C211, was used. This mutant exhibits more glucosyltransferase activity, greater adherence to glass in vitro, and more virulence in gnotobiotic rats than the parent strain (20, 26). Stock cultures were maintained (4°C) in brain heart infusion agar stabs that contained excess calcium carbonate. Cultures of this bacterium were grown in brain heart infusion broth (37°C, 18 h) in an atmosphere of 95% nitrogen and 5% carbon dioxide immediately before infection of animals. Bacterial immunogen was prepared from cultures of *S. mutans* grown in dialyzed media (37°C, 18 to 24 h [18]).

Formalin-killed *S. mutans* 6715, C211 antigen was prepared as previously described (20, 22). One portion of the killed cells was washed (five times) in saline (10,000 × *g*, 30 min), suspended in 0.1% Formalin-saline, and used as antigen for oral immunization of COBS/CD rats. The remaining killed cells were washed in sterile, distilled water (five times), lyophilized, and used as antigen for oral immunization of germfree rats. The number of equivalent colony-forming units (CFU) per ml was determined spectrophotometrically. A standard curve was established from 10 separate determinations of the absorbance at 660 nm of the bacterial suspension versus the number of *S. mutans* 6715, C211 CFU grown anaerobically (95% nitrogen and 5% carbon dioxide) at 37°C on blood agar and mitis-salivarius agar.

Rats were also challenged with additional antigen preparations including heat-killed *S. mutans* 6715, C211 (5×10^8 equivalent CFU per ml) for intravenous (i.v.) immunization and five-times-washed (saline) sheep erythrocytes (SRBC; 50% suspension) for intraperitoneal (i.p.) immunization.

Experimental design. Weanling (age 20 days) specific pathogen-free COBS/CD rats (25 rats per group) were provided drinking water that contained either 10^7 (group A), 10^8 (group B), or 10^9 (group C) equivalent CFU per ml and diet #305 (23, 28) ad libitum (Fig. 1). Control offspring (groups D and E, 20 rats per group) were given sterile drinking water. At age 90 days, all rats were mated, and, subsequent to parturition, litters were reduced to eight pups per dam. Colostrum, milk, serum, and saliva were collected from each rat dam at 2- to 5-day intervals throughout lactation (17) and assayed for immunoglobulin levels and agglutinin titers to *S. mutans*.

Germfree weanling rats (age 20 days) were transferred from the isolators to sterile, hooded cages maintained in a horizontal, double-sided, laminar air-flow unit (Germfree Laboratories, Inc.). Groups of rats (60 animals per group) were provided diet #305 containing antibiotics (21) and either 10^7 (group A), 10^8 (group B), or 10^9 (group C) equivalent CFU per g of diet (10 mg, 100 mg, 1 g of lyophilized whole cells per kg of diet, respectively) and sterile drinking water ad libitum. The appropriate final concentration of antibiotics and lyophilized *S. mutans* whole cells was initially blended with the vitamin mixture and then extensively mixed with the other dietary ingredients according to the procedures described by Navia (28). Germfree rats (groups D and E) were provided diet #305 with anti-

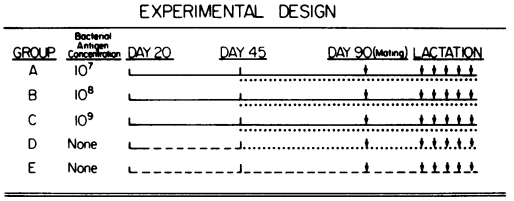

FIG. 1. *Experimental design used in studies of antigen-dose dependence for induction of a secretory immune response. COBS/CD rats provided S. mutans antigen (concentration designated is in equivalent CFU per ml) in drinking water (——); rats mated or days of colostrum, milk, saliva and serum collection (↓); all rats were provided diet #305. Gnotobiotic rats provided S. mutans antigen (concentration designated is in equivalent CFU per gram) in food (——); rats sacrificed, mated, or days of colostrum and milk collection (↓); all rats were provided diet #305.*

biotics and sterile drinking water. Rats in groups A through D (age 45 days) were challenged orally with 50 μl of an 18-h broth culture of *S. mutans* 6715, C211 (5.4×10^6 to 5.8×10^6 CFU). Rats in Group E were uninfected controls. The day after challenge, individual oral swabs were taken and cultured on blood (brain heart infusion broth base) and mitis-salivarius agar to confirm colonization of mutant C211. The presence of this bacterium was confirmed in each experimental animal at weekly intervals throughout the entire test period. No other bacterial species was detected.

Some of the animals (35 to 50) from each group were removed from the experiment at age 90 days. Before sacrifice, saliva and serum were collected from each animal. After sacrifice, the mandibles from each rat were aseptically removed and then defleshed with a sterile scalpel. The right mandible from each animals was stained and scored for the level of plaque (24). The left mandible was used for microbial analyses (25). Subsequent to plaque and microbial analyses, both mandibles from each animal were stained and scored for caries by the method of Keyes (13). The remaining rats (10 to 25) in each group were mated, and, after parturition, samples of colostrum and milk were collected from each rat dam at 2- to 5-day intervals throughout lactation (17).

At the end of lactation, the lateral tail veins of the rats (10 to 15 per group) were injected weekly with heat-killed *S. mutans* 6715, C211 (5×10^8 equivalent CFU per ml) as follows: week 1, 0.75 ml; week 2, 1.5 ml; week 3, 3.0 ml. One week after the last injection, rats were test bled from the lateral tail vein, and each animal was subsequently injected i.p. with a 50% suspension of SRBC (1.0 ml). Four days after injection, rats were exsanguinated by cardiac puncture. All serum samples were collected and assayed for antibody activity to *S. mutans* 6715, C211 and to SRBC.

Chromatographic fractionations, determinations, and antibody assays. Colostrum and milk samples from gnotobiotic animals were centrifuged at $20,000 \times g$ for 2 h at 4°C, and whey was collected from between the insoluble material and lipid layer and decaseinated (19). A portion (1.0 ml) of each colostrum (five to eight per group) and milk (five to eight per group) whey sample was chromatographed on Sephadex G-200 columns (1.6 by 100 cm, Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) equilibrated in 1% ammonium bicarbonate buffer (pH 7.0). Peak fractions were pooled according to their immunoglobulin content and concentrated by negative-pressure dialysis to the original sample volume.

The levels of immunoglobulin in saliva, serum, whey, and whey column fractions were determined by a radial immunodiffusion technique, using anti-rat γ, μ, and α heavy-chain sera and serum and colostral whey standards (17).

All serum, saliva, and whey samples as well as whey fractions from G-200 were assayed for agglutinin activity to *S. mutans* by the microtitration technique (3). Saliva and whey fractions from gnotobiotic rats were assayed for antibody activity to *S. mutans* before and after enhancement with optimum concentrations of either anti-rat α or anti-rat γ heavy-chain sera (17, 20). The levels of anti-SRBC antibodies in sera of immunized rats were determined by microagglutination.

Caries evaluation. Individual pairs of mandibles were cleaned and then stained with murexide. After staining, the molars were hemisectioned with the aid of a dental drill; buccal, sulcal, and proximal caries were scored by the method of Keyes (13). The caries scores from each group of rats were statistically reduced by computing means, standard deviations, and standard errors. Differences among means were evaluated by an analysis of variance and by multiple-mean comparisons, using the Duncan test (5).

RESULTS

Antibody response to *S. mutans* in COBS/CD rats. Low titers of agglutinin activity to *S. mutans* were detected in serum samples from COBS/CD rats (provided *S. mutans* antigen in their drinking water) and their controls (Table 1). Rats provided antigen in their drinking water at a concentration of either 10^8 (group B) or 10^9 (group C) equivalent CFU per ml had high levels of antibody to *S. mutans* in their saliva. The antibody levels of groups B and C were not significantly different. Saliva of rats provided the lowest antigen concentration (group A), and their controls exhibited low agglutinin activity. Similar patterns of agglutinin activity were observed in colostrum and milk from these five groups of animals. The level of antibodies in the milk of rats in groups B and C decreased slightly during lactation; however, the activity present in milk collected between days 15 and 20 was significantly higher than that observed in colostrum of animals in groups A, D, and E. Although colostrum and milk from rats in group A exhibited higher agglutinin titers than those from the infected controls (group D), the values were not significantly different.

Serum and saliva immunoglobulin levels and agglutinin titers. Each of the major classes of immunoglobulins (IgA, -G, and -M) was detected in sera of the five groups of gnotobiotic rats, and no significant differences in amounts could be discerned (Table 2). Low or undetectable levels of antibodies to *S. mutans* 6715, C211 were observed in sera of these animals. Although similar levels of salivary IgG but no IgM (as reported previously; 22) were observed in the five groups, rats provided either 10^7 or 10^8 *S. mutans* CFU per g of diet (groups A and B) displayed approximately three- to fivefold higher levels of salivary IgA than rats provided the highest antigen concentration (group C) and controls (groups D and E). No significant difference was observed in the levels of salivary IgA of groups C, D, and E animals. Saliva of orally immunized animals in groups A and B exhibited significantly higher levels of

TABLE 1. *Agglutinin titers in serum, saliva, colostrum, and milk of COBS/CD rats provided increasing amounts of S. mutans 6715, C211 in their drinking water*

Group	Concn of immunogen[a]	Agglutinin titer[b]						
		Serum	Saliva	Colostrum and milk (days of lactation)				
				1-3	4-6	7-9	11-12	15-20
A	10^7	0.3 ± 0.1	0.8 ± 0.2	2.2 ± 0.3	1.8 ± 0.2	1.7 ± 0.5	2.0 ± 0.4	1.2 ± 0.3
B	10^8	0.3 ± 0.1	3.8 ± 0.2	5.8 ± 0.5	5.1 ± 0.5	4.9 ± 0.3	4.0 ± 0.3	3.8 ± 0.1
C	10^9	0.3 ± 0.2	3.5 ± 0.1	4.5 ± 0.5	4.0 ± 0.2	4.3 ± 0.2	3.0 ± 0.1	3.2 ± 0.3
D	None	0.2 ± 0.1	0.9 ± 0.1	1.3 ± 0.3	1.1 ± 0.2	0.8 ± 0.1	1.1 ± 0.2	0.6 ± 0.3
E	None	0.3 ± 0.1	0.3 ± 0.2	0.4 ± 0.1	0.2 ± 0.1	0	0	0

[a] Each group of rats (12 to 15 per group) was provided Formalin-killed *S. mutans* 6715, C211 whole cells in drinking water. Concentration is in equivalent CFU per milliliter of water. Group D, infected only; group E, noninfected control.

[b] Mean values expressed as $\log_2$ titer ± standard error.

TABLE 2. *Immunoglobulin levels and agglutinin titers in serum and saliva of gnotobiotic rats given increasing amounts of S. mutans 6715, C211 antigen in food and controls*

Group	Concn of immunogen[a]	Serum				Saliva				
		Immunoglobulin[b]			Agglutinin titer[c]	Immunoglobulin[b]		Agglutinin titer[c]		
		IgM	IgG	IgA		IgG	IgA	Untreated	Anti-α	Anti-γ
A	10^7	0.25 ± 0.01	0.88 ± 0.05	0.08 ± 0.01	1.5 ± 0.2	0.14 ± 0.01	0.36 ± 0.05	3.2 ± 0.2	5.7 ± 0.4	2.9 ± 0.2
B	10^8	0.27 ± 0.02	0.73 ± 0.03	0.08 ± 0.01	1.1 ± 0.3	0.13 ± 0.01	0.27 ± 0.02	3.6 ± 0.2	6.0 ± 0.3	2.8 ± 0.2
C	10^9	0.24 ± 0.01	0.74 ± 0.05	0.08 ± 0.01	0.5 ± 0.2	0.10 ± 0.02	0.10 ± 0.01	0.9 ± 0.2	1.5 ± 0.5	1.3 ± 0.4
D	None	0.25 ± 0.01	0.73 ± 0.04	0.07 ± 0.01	0.9 ± 0.2	0.08 ± 0.01	0.08 ± 0.01	0.3 ± 0.2	0.2 ± 0.2	0.1 ± 0.1
E	None	0.23 ± 0.05	0.67 ± 0.03	0.07 ± 0.01	0	0.08 ± 0.01	0.06 ± 0.01	0	0	0

[a] Each group of rats (21 to 24 per group) was provided Formalin-killed *S. mutans* 6715, C211 whole cells in their diet. Concentration is in equivalent CFU per gram of diet. Group D, infected only; group E, noninfected control.

[b] Mean values expressed in milligrams per milliliter ± standard error. No IgM was detected in saliva.

[c] Mean values expressed as $\log_2$ titer ± standard error. Agglutinin titer after addition of either anti-rat-α or -γ heavy-chain sera.

anti-*S. mutans* antibody than did saliva of group C rats and controls. This agglutinin activity was enhanced after the addition of optimum concentrations of anti-rat α heavy-chain sera. No augmentation in activity was observed after the addition of anti-rat γ heavy-chain sera. Although group C rats displayed higher agglutinin titers in saliva than did controls, the values were not significantly different.

Colostral and milk whey immunoglobulin levels and agglutinin titers. Whey of gnotobiotic rats obtained during the first 3 days of lactation had more IgA and -G than whey samples obtained after day 4; no immunoglobulin could be detected (Table 3). The levels of IgA in colostral whey of orally immunized rats in groups A and B were two- to threefold higher than those in group C rats and controls, whereas an approximately twofold difference was observed in the level of IgA in milk obtained from these animals. This increased level of IgA in whey correlated with significant agglutinin activity to *S. mutans* 6715, C211. Support that this antibody was of the IgA class was found in the observation that all activity and IgA were associated with the fraction of whey eluting in the first peak of a G-200 column. This agglutinin activity was enhanced after the addition of anti-rat α sera. No augmentation of agglutinin activity was observed after the addition of anti-rat γ sera. Low or undetectable agglutinin activity was observed in whey of rats fed 10^9 equivalent *S. mutans* CFU per g of diet and in control animals.

Effect of oral ingestion of high concentrations of *S. mutans* antigen on induction of a secretory immune response. Because rats fed the highest concentration of *S. mutans* antigen (group C) did not manifest a secretory immune response as demonstrated by the presence of specific IgA antibodies in external secretions (Tables 2 and 3), the levels of anti-*S. mutans* and anti-SRBC antibodies were determined in sera of rats after peripheral immunization with the respective antigen (Table 4). Levels of antibodies to either *S. mutans* or SRBC were either low or undetectable in sera of all five groups of rats before i.v. and i.p. injection of *S. mutans* 6715, C211 and SRBC, respectively.

TABLE 3. *Immunoglobulin levels and agglutinin titers in colostrum and milk of gnotobiotic rat dams fed increasing amounts of S. mutans 6715, C211*

Group	Concn of immunogen[a]	Colostrum (days 1-3)					Milk (days 5-19)				
		Immunoglobulin[b]		Agglutinin titer[c]			Immunoglobulin		Agglutinin titer		
					G-200					G-200	
		IgG	IgA	Whole	Fraction one	Fraction two	IgG	IgA	Whole	Fraction one	Fraction two
A	10^7	0.23 ± 0.13	1.43 ± 0.21	8	8 (11,7)	2 (3,2)	0.17 ± 0.02	0.70 ± 0.08	6	7 (9,5)	1 (1,0)
B	10^8	0.22 ± 0.10	1.39 ± 0.12	7	8 (11,8)	1 (1,0)	0.19 ± 0.03	0.61 ± 0.05	5	5 (7.2)	0 (0,0)
C	10^9	0.20 ± 0.10	0.64 ± 0.10	3	2 (2,0)	0 (0,0)	0.16 ± 0.02	0.40 ± 0.06	1	0 (0,0)	0 (0,0)
D	None	0.25 ± 0.15	0.54 ± 0.15	1	1 (2,1)	0 (0,0)	0.15 ± 0.06	0.45 ± 0.10	0	0	0
E	None	0.20 ± 0.10	0.61 ± 0.15	0	0	0	0.12 ± 0.03	0.30 ± 0.10	0	0	0

[a] Groups of rat dams (five to eight per group) were provided Formalin-killed *S. mutans* 6715, C211 whole cells in their diet. Concentration is in equivalent CFU per gram of diet.

[b] Mean values expressed in milligrams per milliliter ± standard error. IgM was not detectable in this secretion.

[c] Value expressed as $\log_2$ titer in pooled whey samples (eight per group). Values in parentheses are $\log_2$ titer after addition of either anti-rat-α or -γ heavy-chain sera, respectively.

TABLE 4. *Response after i.v. injection of S. mutans antigen and i.p. injection of SRBC*

Group	Immunogen		Serum agglutinin titer[c]	
	Food ag level[a]	2°C challenge[b]	Post C211	Post SRBC
A	10^7	C211 (i.v.)	9.5	8.1
		SRBC (i.p.)	0	7
B	10^8	C211 (i.v.)	9.8	8.4
		SRBC (i.p.)	0	6.7
C	10^9	C211 (i.v.)	10.4	7.5
		SRBC (i.p.)	0	6
D	None	C211 (i.v.)	9.3	10
		SRBC (i.p.)	0	6.5

[a] Each group of rats (21 to 24 per group) was provided Formalin-killed *S. mutans* 6715, C211 whole cells in their diet. Concentration is in equivalent CFU per gram of diet. Group D, infected only. ag, Antigen.

[b] Animals received weekly injections of heat-killed *S. mutans* 6715, C211 according to the following schedule: week 1, 0.75 ml; week 2, 1.5 ml; week 3, 3.0 ml. One week after the last injection, each animal was challenged i.p. with 1.0 ml of a 50% SRBC suspension.

[c] Mean values expressed as $\log_2$ titer ± standard error. Agglutinin titer after addition of either anti-rat-α or -γ heavy-chain sera.

However, after secondary challenge with C211 and SRBC, significant agglutinin activity was detected in the sera of all of the animals from the four test groups (A through D).

Plaque and microbial analyses. The levels of plaque on mandibular molars of orally immunized animals (groups A and B) were significantly lower ($P \leq 0.05$) than those observed in group C and infected-control rats (Table 5). Although the levels of plaque in group C rats, which received *S. mutans* antigen, were lower than the infected controls, the values were not significantly different. The amount of viable *S. mutans* in plaque of group A rats was lower than in plaque of group B animals. These values were significantly lower than the amount of viable *S. mutans* in plaque of groups C and D. Germfree rats (group E) exhibited no plaque or viable *S. mutans*.

TABLE 5. *Levels of plaque and numbers of S. mutans 6715, C211 in plaque of 90-day-old gnotobiotic rats provided increasing amounts of S. mutans antigen in food*

Group	Concn of immunogen[a]	Plaque score[b]	No. of viable *S. mutans* 6715, C211 (CFU × 10^5)[c]
A	10^7	7.4 ± 0.5	0.9 ± 0.4
B	10^8	7.0 ± 0.4	3.8 ± 0.6
C	10^9	12.6 ± 0.4	16.8 ± 5.0
D	None	15.1 ± 0.9	17.0 ± 1.8
E	None	0.0	0.0

[a] Groups of rats (35 to 50 per group) were provided Formalin-killed *S. mutans* whole cells in their diet. Concentration is in equivalent CFU per gram of diet. Group D, infected only; group E, noninfected control.

[b] Represents mean value of smooth surface plaque on one mandible per rat per group ± standard error.

[c] Represents mean number of CFU in plaque from one mandible per rat per group (as determined on blood and mitis-salivarius agar) ± standard error.

Immune protection. Rats that exhibited high levels of salivary IgA antibodies to *S. mutans* were protected from caries formation when challenged with live, virulent *S. mutans* 6715, C211 (Table 6). Rats in group A had fewer carious lesions on buccal, sulcal, and proximal molar surfaces than rats fed a diet containing a 1-log-higher concentration of immunogen. However, both groups exhibited significantly fewer carious lesions on all molar surfaces than animals receiving 10^9 CFU per g of diet. Although the levels of caries in group C rats were lower than those of infected controls, both groups had

TABLE 6. *Mean caries scores from 90-day-old gnotobiotic rats provided increasing amounts of S. mutans 6715, C211 antigen in food*

Group	Concn of immunogen[a]	Caries score[b]						Mean body wt[c]
		Buccal		Sulcal		Proximal		
		Enamel	Dentinal (slight)	Dentinal (slight)	Dentinal (moderate)	Enamel	Dentinal (slight)	
A	10^7	7.6 ± 0.3	5.8 ± 0.1	3.4 ± 0.3	2.1 ± 0.3	0.5 ± 0.2	0.0	233.3 ± 13.7
B	10^8	9.5 ± 0.2	7.0 ± 0.3	4.8 ± 0.4	3.0 ± 0.4	1.6 ± 0.3	0.5 ± 0.2	226.6 ± 5.6
C	10^9	19.3 ± 1.0	17.1 ± 1.0	17.7 ± 1.1	15.8 ± 1.2	7.0 ± 0.2	5.0 ± 0.4	221.7 ± 13.2
D	None	23.8 ± 0.6	18.1 ± 0.9	20.1 ± 0.7	18.1 ± 1.0	7.5 ± 0.3	6.2 ± 0.4	221.1 ± 15.0
E	None	0.0	0.0	0.0	0.0	0.0	0.0	225.6 ± 8.9

[a] Groups of rats (35 to 50 per group) were provided Formalin-killed *S. mutans* whole cells in their diet. Concentration is in equivalent CFU per gram of diet. Group D, infected only; group E, noninfected control.

[b] Evaluated by the method of Keyes (13). Values represent mean ± standard error of caries on the mandibular molar surfaces.

[c] Expressed in grams ± standard error.

extensive decay, and no significant difference was observed. No differences were observed in the mean body weights of any of the groups tested (A through E).

DISCUSSION

Previous investigators have indicated that the induction of a salivary sIgA immune response, after either local injection of *S. mutans* antigen (either whole cell [18, 33] or glucosyltransferase enzyme preparation [34]) or oral ingestion of *S. mutans* whole-cell antigen (22), correlates with protection against dental caries in rodents. The present study suggests that the selective induction of a secretory immune response (after oral ingestion of *S. mutans* whole-cell antigen) is dependent upon the antigen dosage. COBS/CD rats provided *S. mutans* 6715, C211 antigen in their drinking water (final concentration, 10^8 to 10^9 equivalent CFU per ml) exhibited significant levels of specific antibodies in saliva and mammary gland secretions. These findings support previous observations concerning gnotobiotic rats that were provided a similar antigen preparation in their drinking water at a concentration of 10^8 equivalent CFU per ml (22). On the other hand, rats provided 10^7 equivalent CFU per ml of drinking water exhibited agglutinin activity to *S. mutans* similar to that observed in control animals. These results would suggest that the concentration of antigen presented to these animals was insufficient to induce a secretory immune response.

The settling of high concentrations of antigen in water presented a problem that was solved by using food as a carrier. A diet containing 10^7 to 10^8 equivalent CFU per g of diet elicited a secretory immune response as evidenced by the finding that treated rats had higher levels of sIgA in saliva (three- to fourfold), colostrum (two- to threefold), and milk (twofold) than did their controls. Furthermore, these animals exhibited significant levels of sIgA antibodies to *S. mutans* in saliva ($\log_2 = 3$ and 4, respectively) and milk ($\log_2 = 6$ and 5, respectively).

Rats provided higher concentrations of antigen in their diet (10^9 equivalent CFU per g) exhibited levels of immunoglobulin and antibody similar to those of controls. Because low levels of antibody were detected in sera of these animals, the results would suggest that even with a high concentration of *S. mutans*, whole-cell antigens did not penetrate the mucosal barrier and subsequently induce a systemic immune response. The low levels of detectable antibody in colostrum, milk, and saliva of these animals suggest the induction of a state of hyporesponsiveness to *S. mutans* antigen in secretory tissues. Recently, André and co-workers (1, 2) reported that intragastric administration of large doses of SRBC to mice rendered them tolerant to subsequent injection of antigen. These animals exhibited circulating antibodies to the antigen, and the authors attributed unresponsiveness to the formation of immune complexes after peripheral administration of SRBC. These results differ from the present findings in two main regards: (i) Antigen administered in a single, large dose resulted in a serum response (2), whereas continual oral feeding of antigen, as reported here, did not induce circulating antibodies. Furthermore, oral administration of bacterial antigen at optimal concentrations resulted in a secretory immune response. (ii) After oral administration of antigen, André and co-workers (2) observed unresponsiveness when antigen was subsequently ingested i.p. However, in the present study, i.v. administration of bacterial antigen

in orally immunized rats resulted in significant titers of serum antibodies. From our studies it would appear that the unresponsiveness that occurred after feeding large doses of *S. mutans* antigen was a manifestation of tolerance only in the secretory immune compartment. Proof of this will require further investigation. Studies along these lines are currently underway.

It is noteworthy that COBS/CD rats did not manifest a secretory immune response when challenged with a low antigen concentration, a dose which elicited a significant response in gnotobiotic rats. This finding could be due to the experimental animal model and/or the method by which the antigen was delivered. In the former case, a lower concentration of antigen may have effectively induced an immune response in germfree animals, since these animals had previously been exposed to only minimum antigenic stimuli, e.g., food antigens. This could also explain the induction of a condition of hyporesponsiveness in gnotobiotic rats when they were provided a concentration of antigen that elicited a significant response in COBS/CD rats. An alternative explanation for this difference in the induction of an immune response after oral administration of various antigen concentrations to COBS/CD and gnotobiotic rats is the method by which the antigen was delivered. Administration of antigen in food may be more effective than water in presenting antigen to the clones of IgA precursor B-cells located in gut-associated lymphoid tissue and a subsequent induction of a selective secretory immune response (10, 19a, 22, 27, 30, 31).

The direct correlation between the induction of a selective secretory immune response and caries immunity was indicated by the demonstration that gnotobiotic rats, which had specific sIgA antibodies in saliva, colostrum, and milk after ingestion of *S. mutans* antigen, had exhibited significantly smaller amounts of *S. mutans* in plaque and lower levels of plaque and caries on all molar surfaces than did the control animals or those rats that received antigen but did not exhibit specific antibodies after oral ingestion of *S. mutans*.

The results of this study clearly demonstrate that the selective induction of a secretory immune response is (i) antigen-dose dependent, and (ii) correlated directly (in gnotobiotic rats) with protection against the disease. However, additional investigations are required to determine the mechanism(s) involved in the selective induction of a secretory immune response, including duration and memory of the immune response, the events that lead to the appearance of sIgA antibodies in external secretions, and how these antibodies affect immune protection.

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